

Original Article

Moderate and high intensity chronic exercise reduces plasma tumor necrosis factor alpha and increases the Langerhans islet area in healthy rats

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Abstract

Objective: This study aimed to examine the effects of moderate (MIT) and high-intensity training (HIT) chronic exercise on plasma tumor necrosis factor alpha (TNF- α) level and its impact on Langerhans islet morphology in healthy rats. **Methods:** Two-month old normal male Wistar rats were divided into three groups: control (C, n=6), MIT (n=6), and HIT (n=4). The training protocol consisted in 24 sessions of running on a treadmill at 60-80% maximal oxygen consumption (VO₂max) for MIT, and >80% VO₂max for HIT. TNF- α and insulin were measured with ELISA tests. Duodenal pancreas was dissected to analyze the Langerhans islets by immunohistochemistry, a correlation analysis was performed with the nuclei/total islet area. **Results:** HIT and MIT rats showed lower TNF- α plasma levels than controls. Plasma insulin level decreased significantly in HIT compared with C and MIT. In addition, the islet area and nuclei density per islet were higher in the exercise groups compared with C. However, none of the groups showed PD 1 immunoreactivity. **Conclusions:** Under healthy conditions, the chronic exercise reduced plasmatic TNF- α level, and in the same sense, increased the size of the Langerhans islets, depending to the exercise intensity.

Keywords: Exercise, Insulin, Langerhans Islets, Pancreas, TNF- α

Introduction

Tumor necrosis factor alpha (TNF- α), a pluripotent cytokine produced by macrophages and adipocytes¹, has

been implicated in the development of insulin resistance^{2,3} through serine phosphorylation located on its tyrosine kinase domain⁴. White adipose tissue (WAT) expresses and releases TNF- α in an amount proportional to WAT. Previous studies have analyzed the negative effects of TNF- α on β -cells in rodents⁵ and humans⁶; TNF- α coupled with IL-6 and IL-1 participates in local inflammatory responses⁷ mediated by specific membrane receptors, TNF-R1 and TNF-R2, once activated, the cytokine leads to apoptotic mechanisms⁸. In addition, it has been reported that inhibition of MAPK activity by TNF- α elicits insulin resistance⁹. In summary, a reduction in insulin transcription⁷ and secretion^{2,10} lead to a greater vulnerability to autoimmune attack¹¹, and to a negative effect on the β -cell proliferative rate⁵.

Regular exercise induces anti-inflammatory effects with elevated levels of anti-inflammatory cytokines and suppression of TNF α ¹². Both high and moderate intensity training reduced the systemic TNF- α and IL-6 levels^{13,14}.

The authors have no conflict of interest.

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Thereby, exercise offers a protection against TNF- α -induced insulin resistance.

The response to exercise greatly depends on training intensity, as it was previously found in protein expression changes after different training regimes^{15,10}.

The aim of the present study was performed to establish the effects of chronic exercise intensity on TNF- α plasma levels after 48 hours of delay using a cytokine concentration assay in healthy adult rats. The effects of TNF- α concentration on islet histology and plasma insulin levels were also analyzed.

Methods

Ethics statement

The experiments were performed in accordance with the Guide of United States National Institutes of Health for the Care and Use of Laboratory Animals. Animal studies and experimental procedures were approved by the Bioethics and Biosecurity Committee of the Medicine Faculty and CUIB from Colima University, Colima, Mexico (No 2012-05).

Animals

Lean male Wistar rats (2 months old, 200-250 g weight) were individually housed in polyethylene cages in an environmentally controlled room (22-24°C) and a 12-h light/dark cycle. The rats were allowed free access to food (Teklad Global Diet: protein/fat/fiber, 18.0%/5.0%/5.0%) and water. The rats were randomly divided into 3 groups: sedentary control (C, n=6), moderate-intensity training (MIT, n=6), and high-intensity training (HIT, n=4). The animals were weighted at the beginning of each week during training period and before each training session in an *ad libitum* state.

Training schedule

All exercise sessions were carried out between 9 AM and 12 PM. The rats were exercised following a previously described protocol¹⁶, with certain modifications. Before the exercise program, all animals underwent a preconditioning running regimen for a week that consisted of 30 min of daily running at a speed of 15 m/min on a rat treadmill (Modular Treadmill Simplex, Mod. 42528; Columbus Instruments, Columbus, Ohio). Mild electric shocks were used to persuade the animals to run, however most animals ran voluntarily during their initial run. The rats that failed to run regularly were excluded from the training protocol. The training protocol was performed over an 8-week period, based on a previous paper describing chronic exercise in Wistar rats¹⁷. Every training session began with a 10 min warm-up (18 m/min), after which the tilt and speed were progressively increased for 60 min to the appropriate speed in each training group (22 m/min- MIT group, or 28 m/min- HIT group); the sessions ended with a 10 min recuperation period (18 m/min). The trained rats ran at a constant inclination angle of 25° because changes in this parameter significantly affect the $VO_2 \max$ ¹⁶. Under these conditions, and based in previous papers¹⁶⁻¹⁸, the training ensured 60-80% of maximal oxygen consumption^{16,18,19}. The entire experiment was completed in 8 weeks.

Body and heart weight measure, and pancreas tissue collection

At the beginning of the exercise regimen, all the rats from each experimental group (C, MIT, and HIT) were tagged and weighted *ad libitum* state. The weight of each rat at the beginning of each week, before the training session, was recorded for the 8 weeks that the chronic exercise regimen lasted. Additionally, to explore possible heart hypertrophy, 4.5 days after the last training session, the heart was removed and weighed, and the value was considered as heart wet weight. The pancreas tissue (duodenal region) was also collected.

Insulin and Tumor Necrosis Factor-alpha (TNF- α) determinations

Forty-eight hours after the last training session and in an *ad libitum* state, 0.5 mL of venous blood were collected via cardiac puncture in all the rats. In these samples, plasma insulin was measured using an ELISA kit (Abcam, ab100578), and TNF- α determinations were carried out with the specific ELISA kit (Thermo SCIENTIFIC, ER3TNFA, Rockford IL.) according to the manufacturer's instructions. Blood samples were stored in Eppendorf tubes with EDTA, centrifuged at 2000 rcf for 20 min, and the supernatant plasma was stored at -80°C for subsequent ELISA assays, according to the manufacturer's instructions. Absorbance was measured at 450 nm (iMark microplate absorbance reader, BIO-RAD, Cat. 168-1130, Hercules, CA) 30 min after stop solution was added. The samples were measured in duplicate, and the mean absorbance was calculated. Detection limits were < 50 pg/mL for insulin and < 15 pg/mL for TNF- α . Inter and intra assay means coefficients of variance were < 10% and < 12% for insulin and < 10% and 10% for TNF- α .

Pancreas dissection and staining

To analyze the chronic exercise effects on Langerhans islets histology and islet area, hematoxylin-eosin staining was used as follows: 4.5 days (*ad libitum*) after the final training session, rats were anesthetized with sodium pentobarbital (2.5 mg/100 g body weight i.p.) to be subjected to a surgical procedure for the dissection and extirpation of pancreatic tissue. Pancreatic tissue, free of fat and connective tissue, was fixed overnight in a freshly prepared 10% buffered formaldehyde solution. The tissue samples were dehydrated with ethanol and embedded in paraffin wax. Sections of 5 μ m were cut on a rotary microtome and deparaffinized at 100°C for 20 min, transferred to xylene, ethanol-xylene, and absolute alcohol, and finally washed in distilled water to be processed. The sections were stained in hematoxylin (6 min), rinsed in running tap water (20 min), distained in acid alcohol, and rinsed in tap water (5 min), and immersed in lithium carbonate (3 sec), rinsed in tap water (5 min), counterstained in eosin (15 sec), dehydrated in ethanol 95% (3 min) (discarding it after each use), treated with ethanol 95% (3 min), ethanol 100% (3 min), ethanol 100% (3 min), and cleared in xylene (5 min). The sections were mounted with Entellan (Merck, Darmstadt).

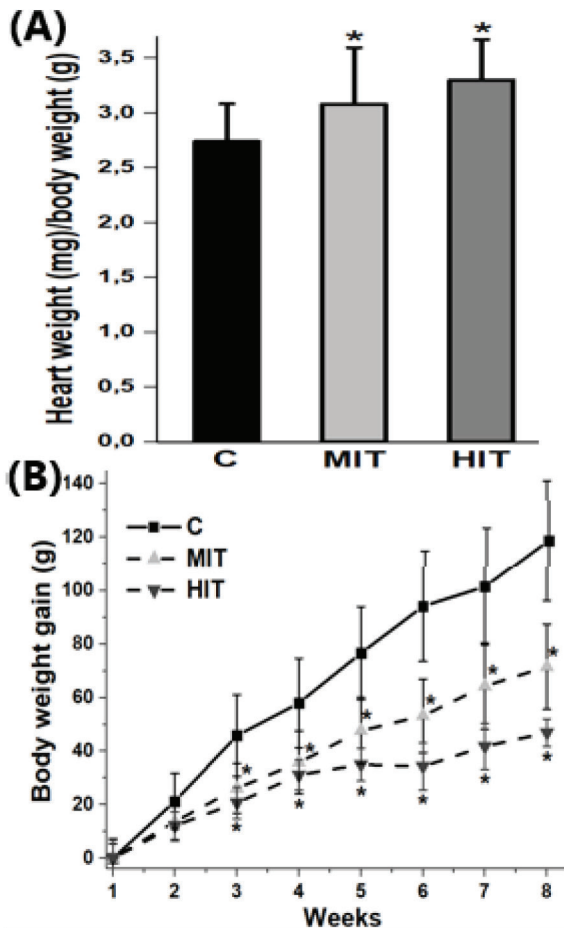


Figure 1. (A) Effects of chronic exercise on heart/body weight ratio, and (B) on body weight gain in adult rats. C, control-sedentary rats; MIT, moderate-intensity exercised rats; HIT, high-intensity exercised rats. Data are expressed as means \pm SD. ANOVA and Dunnett's post hoc * $p=0.03$ MIT and * $p=0.008$ HIT vs. C.

Islet area measurement and nuclei count

Photomicrographs were taken with an Axiocam MRC-5 model digital camera (Carl Zeiss) attached to an AxioPlan 2M model bright-field optical microscope (Carl Zeiss) with a motorized stage and A-plan 10X, and 20X objectives (total magnification, 100X and 200X respectively). Using the MosaiX and Autofocus modules, images of the entire sample surfaces were scanned, and the islet areas were measured by outline spline. All images were obtained with the same lighting conditions and exposure times in the AxioVs 40 V.4.7.0.0 imaging program (Carl Zeiss Imaging Solutions GmbH, 2006-200 imaging program). Axiovision 4.7 (Carl Zeiss Imaging Solutions GmbH, 2006-200 imaging program) software was used to measure the islet area. The nuclei (purple stained) in the islets were counted manually with NIH ImageJ software.

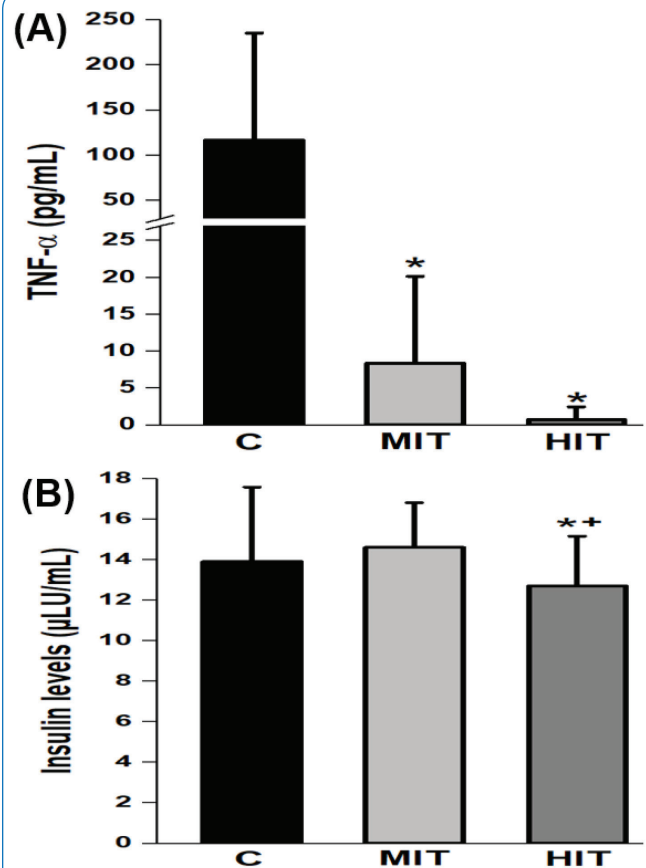


Figure 2. (A) Effects of chronic exercise on plasma TNF- α concentration (pg/mL), and (B) on plasma insulin concentration in adult rats. C, control-sedentary rats; MIT, moderate-intensity exercised rats; HIT, high-intensity exercised rats. Data are expressed as mean \pm SD, ANOVA, and Dunnett's post hoc; * $p=0.02$ HIT vs. C. * $p=0.003$ HIT vs. MIT.

Statistical analysis

Numerical results were expressed as mean \pm standard deviation (SD). All statistical analyses were performed using the SPSS 17.0 statistical software package. Data of experimental groups were compared in a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test; a value of $p < 0.05$ indicated statistical significance. Correlation analysis (linear regression) was performed in individual groups to evaluate the association between variables (nuclei number and islet area).

Results

Cardiac hypertrophy

As cardiac hypertrophy is considered an adaptation mechanism to exercise²⁰, the heart/body weight ratio in these experiments, was evaluated. Figure 1A shows that MIT and HIT groups developed cardiac hypertrophy. The ratio in

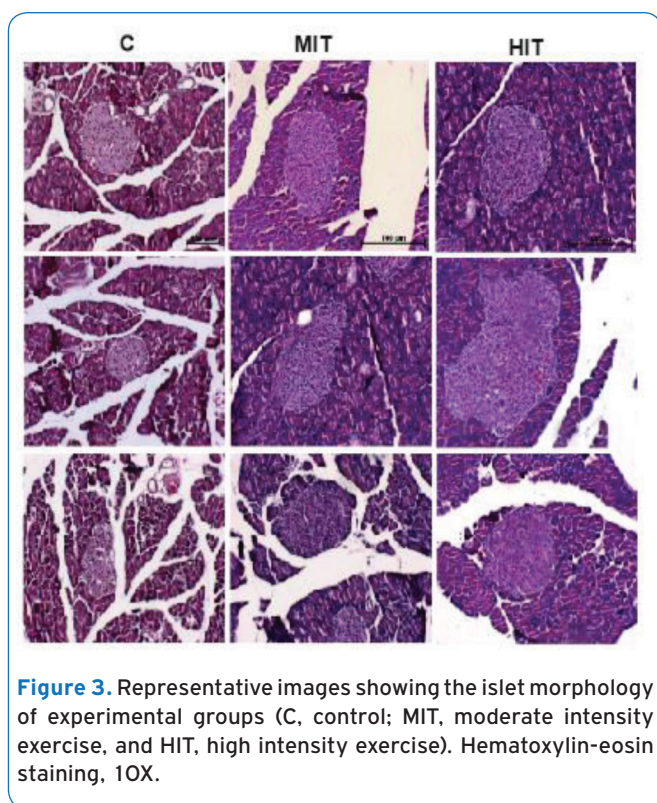


Figure 3. Representative images showing the islet morphology of experimental groups (C, control; MIT, moderate intensity exercise, and HIT, high intensity exercise). Hematoxylin-eosin staining, 10X.

the C and MIT groups was 2.7 ± 0.34 and 3.08 ± 0.51 ($p=0.03$ vs. C), whereas the value in the HIT group was 3.3 ± 0.37 ($p=0.008$ vs. C). Considering these data, we were sure that exercise protocol (intensity and frequency training) was adequate for inducing adaptive response.

Body weight gain decreased in response to chronic exercise

The initial weight in the three groups of rats was similar. The mean values were 237.5 ± 31.9 g, 239.4 ± 11.8 g, and 247.6 ± 15.4 g for the C, MIT, and HIT groups, respectively. The body weight observed during the training program was significantly greater in the C group, compared with the MIT ($p=0.003$) or HIT groups ($p=0.005$) (Figure 1B). These data showed that our exercise protocol led to an overall decrease in weight gain as compared to the control group and this effect was dependent of exercise intensity.

Effects of chronic exercise on plasma TNF- α and insulin

In relation to TNF- α , chronic exercise reduced the pro-inflammatory cytokine concentration. The levels in the C group were 117 ± 118.1 pg/mL, whereas that in the MIT and HIT groups were 8.4 ± 11.8 pg/mL and 0.71 ± 0.17 pg/mL, respectively ($p=0.04$ vs. C for both groups) (Figure 2A). Particularly in the HIT group, some rats had lower concentrations of TNF- α , and the ELISA kit sensitivity was unable to detect this cytokine.

Previous studies conducted in HIT-T15 pancreatic β -cell

line culture indicates a negative effect of TNF- α on glucose-stimulated insulin secretion^{2,7}. The benefits of exercise on insulin resistance in type 2 diabetes mellitus patients are also associated with decreased plasma TNF- α concentration¹⁰. In our experimental groups, plasma insulin levels measured after chronic exercise did not change significantly in the MIT group compared with the C group ($p=0.11$); however, insulin levels in HIT group decreased significantly compared to C and MIT groups ($p=0.02$ and $p=0.0003$, respectively) (Figure 2B); the values were 13.9 ± 3.7 μ IU/mL for the C group, 14.6 ± 2.2 μ IU/mL for MIT group, and 12.7 ± 2.5 μ IU/mL for HIT group. These data indicate that changes in TNF- α levels induced by chronic exercise only affected plasma insulin levels in HIT group.

Effects of chronic exercise on the islet area and cell number per islet

Figure 3 shows optical micrographs of Langerhans islets for C, MIT, and HIT groups. The histological analysis did not reveal morphological differences in the islet of Langerhans between the three groups studied. Pancreatic islets did not show pyknotic nuclei and infiltration (Figure 3). Moreover, in the three groups, the pancreatic islets had a regular oval profile that reflected normal islets²¹. Finally, in all groups, the pancreatic islets showed a smooth counteracted perimeter with β -cells centrally enveloped by other non β -endocrine cells that represent normal islets²². To better understand the TNF- α changes on the pancreatic mass, immunohistochemistry was performed to determine the presence of receptor programmed cell death 1 (PD1) (1:200 Cell Marque) which is positively regulated by TNF- α ²³. No immunoreaction for PD1 was found in the groups of rats studied (supplementary figure). To discard an error during the immunostaining process, the staining was done in parallel with a positive control tissue (tonsil); in this tissue, positive staining showed a clear immunoreaction (supplementary figure, +Tissue). Figure 4A shows the total islet area in all the experimental groups. In detail, the islet area for C was 11935 ± 11054 μ m²; in MIT the islet area was 20807 ± 14198 μ m² ($p=0.01$ vs C); in HIT the islet area mean was 30783 ± 21982 μ m² ($p=0.0001$ vs C, and $p=0.01$ vs MIT). Figure 4B shows the nuclei number per islet; for C group the value was 101.3 ± 81.41 . The exercised rats to moderate intensity, the mean of nuclei number was 145.9 ± 85.58 ($p=0.01$ vs C); finally, the total nuclei per islet for HIT were 178.3 ± 129.1 ($p=0.003$ vs C). A correlation analysis between the nuclei number/islet area was carried out for each rat; Figure 4C shows a significant correlation in all the groups is showed: in C, $r^2=0.92$ ($p=0.0008$); in MIT, $r^2=0.82$ ($p=0.0009$); and in HIT $r^2=0.86$ ($p=0.0009$). Considering these results, this study showed a reduction in TNF- α levels in HIT rats, accompanied by a higher nuclei density per islet, at least in a healthy state.

Discussion

The present study showed a lower weight gain after chronic exercise in MIT and HIT groups, in comparison to a

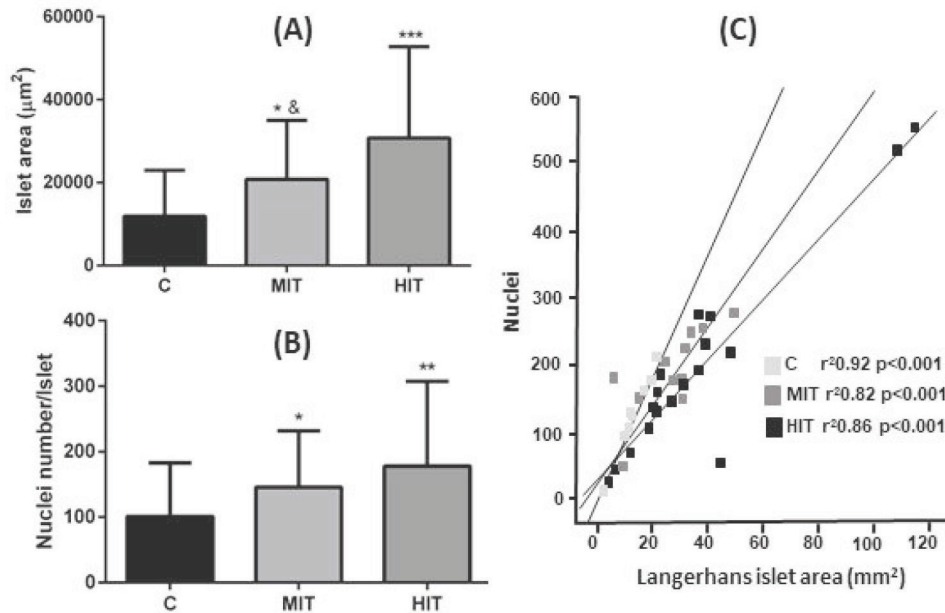


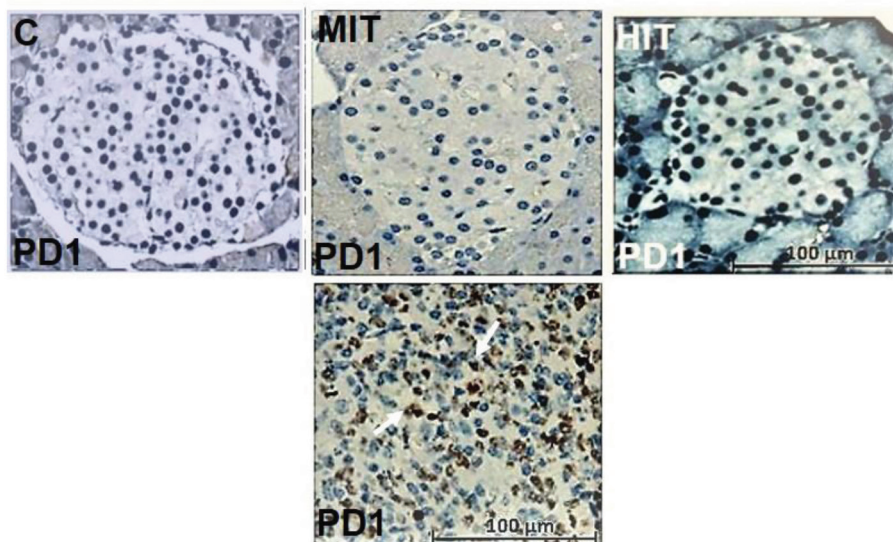
Figure 4. (A) Effects of chronic exercise on total islet area, (B) nuclei number per islet, and (C) correlation between nuclei and Langerhans islet area in adult rats. C, control-sedentary rats; MIT, moderate-intensity exercised rats; HIT, high-intensity exercised rats. Data are expressed as means \pm SD, ANOVA and Dunnett's post hoc * $p < 0.05$ vs. C; & $p = 0.01$ vs. HIT; ** $p = 0.003$ vs. C and *** $p = 0.0001$ vs. C.

previous report²⁴. However, another study has also shown a lack of weight change after slow chronic exercise²⁵. Because the running velocity training used in our study (22 and 28 m/min) was closer to that in the first study mentioned²⁴ we believe that training intensity plays an important role in attenuating weight gain.

The TNF- α levels in plasma were lower after chronic exercise in MIT and HIT groups (Figure 2A). It should be mentioned that some rats in the HIT group had undetectable levels of TNF- α , as was previously shown in healthy Wistar rats²⁶, where a delayed time in blood sampling (48 hours) was used. Given that WAT is considered the principal source of TNF- α in a chronic systemic inflammation²⁴, non-obese healthy rats have less WAT tissue and systemic TNF- α concentration, compared with obese animals²⁴. The slower weight gain observed in our trained groups might be associated with a lower amount of WAT in MIT and HIT groups, and could result in a lower systemic concentration of TNF- α (Figure 2A). On the other hand, acute running exercise does not change the systemic TNF- α levels in diabetic rats²⁷, as occur in obese subjects²⁸; training resistance (a model whose effects are opposite to those of the aerobic running exercise) did not change the TNF- α levels compared with controls rats²⁹. However, chronic resistance training increases systemic TNF- α levels in diabetic male rats³⁰. When swimming is used as a training exercise, TNF- α levels decrease, and pancreas weight is maintained¹⁴. Therefore, these results indicate that aerobic exercise is anti-inflammatory in nature and constitute a good training protocol to reduce plasma TNF- α

concentration in a healthy state. Nevertheless, this last statement does not apply to diabetic models³¹. Length of training seems to play a key role in systemic TNF- α down-regulation, and three weeks of running (shorter time than ours) did not reduce plasma TNF- α in a healthy state³², as occurred with acute aerobic exercise²⁹. Moderate physical exercise has marked anti-inflammatory effects on diabetic rats and is used as a strategy to protect diabetics from physiopathological complications³². Exercise training enhanced the ability of isolated adipocytes to secrete TNF- α to reduce sTNFR1 receptor secretion; this alteration may enhance the autocrine effects of TNF- α within adipocytes in exercise-trained rats²⁶. Our results showed that systemic TNF- α level was lower in the training states (MIT and HIT) compared with a sedentary condition (control) (Figure 2A), parallel results with those obtained in HIT group were showed plasmatic insulin that also decreased significantly (Figure 2B). These data suggest a higher insulin sensitivity for HIT compared with C and MIT groups as was seen in islet morphology (Figure 3). Latter results coincide with the results obtained in studies reporting negative effects of TNF- α on Langerhans islets in diabetic models^{3,6,14}; the lower grades of the inflammatory condition in these rats change the micro-environment of the Langerhans islets and allow them to produce insulin for longer periods, a key role in islet physiology⁶.

In fact, data of current study indicated that there is a threshold in the TNF- α concentration to significant impact to insulin levels (fig 2B). The lack of immunoreactivity for the apoptosis marker, PD1, a protein activated by TNF- α ²³,



Supplementary Figure. Representative images that show the lack of PD1 staining in the Langerhans islets in all groups studied (C, MIT, HIT, as in Figure, 3), and in a positive tissue (tonsil). Note the clear immunostaining for PD1 in the positive tissue, some of them indicated by white arrows, 20X.

in the C group supports our hypothesis (supplementary Figure). The islet area increased in MIT and HIT groups was accompanied with a higher nuclei density/islet (Figure 4A and Figure 4B). To our best knowledge, this is the first work to report the impact of the exercise intensity on the islet area in healthy rats. It is known that the chronic exercise increases the circulating growth factor levels such as IGF-1^{33,34}, that positively impact in the beta cells mass³⁵. We do not discard the possibility that the training protocol applied in this paper could be a sufficient stimulus to elevate circulating IGF-1, and positively impact the islet area and nucleus density.

Conclusion

Moderate and high intensity chronic exercise performed in healthy rats reduced the levels of the pro-inflammatory cytokine TNF- α in basal conditions, depending on exercise intensity. The experiments of the current study also suggest the presence of a threshold of systemic TNF- α level to significantly impact insulin concentration in mentioned rats. As the samples were collected 48 hours after finishing the training program, the results obtained were not a consequence of the last exercise session, but to a chronic long-term exercise intensity depending .

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Author contribution

AJM, SM, ERAB and AVO conceived the idea and designed the project; AJM, ML, SM performed the experiments; VM, AJM, ARH, SM, JM, ERAB interpreted the results of the experiments; AJM, ERAB, AVO drafted the manuscript; AJM, ERAB, AVO, SM, edited and revised de manuscript; VM, ERAB, VM, AGD, JCC, contributed reagents, materials analysis tools; VM photographic work; AGD, ARH, MAM histological work.

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